First observation of intersex in *Trachurus trachurus* (Carangidae) from the Eastern Coast of Algeria: are nematodes the causative factor?

by

Keltoum ICHALAL* (1, 2), Zouhir RAMDANE (2), Mokrane IGUER-OUADA (1)
& Mohammed KACHER (3)

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Abstract. – Intersexuality was reported at macroscopic and microscopic levels in horse mackerel, *Trachurus trachurus* (Linnaeus, 1758) from Algerian coast. Macroscopically, intersex specimens were characterized by the simultaneous presence of both male and female gonads. Gonads were typical ovaries with a testicular component embedded in one of the two ovaries. Histological analysis revealed the simultaneous presence of female and male germ cells. Intersex was also observed, but exclusively at microscopic level, in normal females with no macroscopic intersex. In these specimens, histological sections showed a predominance of female tissue with oocytes at different developmental stages. Male tissue was characterized by seminiferous tubule-like structures with a predominance of spermatogonia compared to spermatocytes and spermatids. No mature spermatozoa were observed. No intersex was observed in males. The observed intersex was considered as masculinisation. The parasitic analysis revealed the presence of both Nematoda and Digenea with P = 31.20%, mI = 2.59 per fish and mA = 0.80, and P = 13.15%, mI = 1.51 per fish and mA = 0.19, respectively. The factors inducing intersex remain unknown; however, the results showed that 100% of intersex females presented a parasitic infestation by Nematoda highlighting the probable impact of these parasites as a causative factor, probably through pollution concentration.

Résumé. – Signalement de premiers cas d'intersexualité chez *Trachurus trachurus* (Carangidae) sur la côte Est algérienne : les nématodes sont-ils l'agent causal ?

Cette étude signale l'existence de l'intersexualité chez les femelles de *Trachurus trachurus* (Linnaeus, 1758) de la côte Est algérienne. Deux femelles ont révélé, à l'échelle macroscopique, la présence simultanée de tissus gonadiques mâles et femelles. Les gonades présentaient des fragments de tissu testiculaire lié intimement au tissu ovarien majoritaire. L'étude histologique a également mis en évidence la présence simultanée de cellules germinales mâles et femelles. L'intersexualité est révélée aussi dans des gonades femelles, mais uniquement à l'échelle microscopique. Dans ce cas, l'histologie a révélé la présence simultanée d'ovocytes à différents stades de développement et d'un tissu mâle caractérisé par des structures similaires aux tubes séminifères avec une prédominance de spermatogonies par rapport aux spermatocytes et spermatides. Aucun spermatozoïde mature n'a été observé. De même, aucune intersexualité n'a été observée chez les mâles, mettant ainsi en évidence la seule masculinisation des femelles. L'analyse parasitaire a révélé la présence de nématodes et de digènes avec P = 31,20% ; mI = 2,59 par poisson et mA = 0,80, et P = 13,15% ; mI = 1,51 par poisson et mA = 0,19, respectivement. Les causes précises de l'intersexualité restent méconnues ; cependant, les résultats ont montré que celle-ci est observée exclusivement chez des femelles parasitées par des nématodes, faisant ainsi ressortir le rôle probable de ces parasites comme facteur causal, notamment par accumulation de la pollution aquatique.

Horse mackerel, *Trachurus trachurus* (Linnaeus, 1758), is of real commercial interest in the Mediterranean Sea (Abaunza *et al*., 2008); it is of major importance in the pelagic and demersal fisheries where annual landings reached 205,807 tons in 2012 (FAO, 2014).

Horse mackerel presents a reproductive strategy by being gonochoric (Gordo *et al*., 2008). Several studies have been dedicated to its fecundity (Van Damme *et al.,* 2005; Gordo *et al.,* 2008), effect of temperature on embryonic development (Cunha *et al.,* 2008), maturity stages (Karlou-Riga and Economidis, 1995; Costa, 2009; Ndjaula *et al.,* 2009), and its parasitism infestations (Gestal and Azevedo, 2005; Pekmezci *et al*., 2012; Mansour *et al*., 2013).

In teleost fish, it is well established that parasitism is associated with a range of disorders affecting life history traits (Bobadilla, 2009; Al-Jahdali and Hassanine, 2010; Ferrer-Maza *et al*., 2014). Reproduction particularly is affected either directly or indirectly, depending on the target organs and the involved parasites (Bobadilla, 2009). Thus, it is reported ovary morphological abnormalities (Itoh *et al*., 2004; Al-Jahdali and Hassanine, 2010) and gonad development delay (Parsakhanghah *et al.,* 2011; Sanoun *et al.,*

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(1) Laboratoire associé en écosystèmes marins et aquacoles (LAEMA), Faculté des sciences de la nature et de la vie, Université de Bejaia (06000), Algérie. [imokrane@gmail.com]

(2) Laboratoire de zoologie appliquée et d'écophysiologie animale, Faculté des sciences de la nature et de la vie, Université de Bejaia (06000), Algérie. [zohir22000@yahoo.fr]

(3) Centre national de recherche et du développement de la pêche et de l'aquaculture, Bousmaïl, Tipaza (42004), Algérie. [mohamed.kacher@gmail.com]

* Corresponding author [ichalalk@gmail.com]

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2012). At microscopic level, parasitism is associated with inflammation, oedema, prenecrotic and necrotic changes with a follicular atresia (Clarke *et al*., 2006). In this respect, Mansour *et al*. (2013) reported in horse mackerel a new species of myxosoan parasite causing oocyte alterations.

Parasitism was also found to be associated with intersex disorders in several animal species such as insects (Vance, 1996), copepods (Ianora *et al.,* 1987), bees (Straka *et al.,* 2011) and amphipods (Rodgers-Gray *et al*., 2004). In fish, it is reported that parasites accelerate sex changes in fish naturally hermaphrodite (Schärer and Vizoso, 2003). Intersex is defined as the presence of both male and female reproductive features within the same gonad (Hinck *et al*., 2009; Barnhoorn *et al*., 2010). Microscopically, intersexuality (ovotestis) is a condition where an individual possesses oocytes and male germinal cells within the gonad of the opposite gender (spermatocytes in the ovary or oocytes in the testis) (Jackson *et al*., 2006).

Although this phenomenon occurs naturally in protandric or protogynous fish species, in gonochoristic species, intersex is not a normal part of the reproductive cycle (Jobling *et al.,* 2002).

The relationships between parasites and their hosts are complex, and neurohormones, hormones and growth factors play prominent roles in these relationships (Jobling and Tyler, 2003). Indeed, it is well established that in vertebrates, parasites may secrete hormones, neuropeptides, or cytokine like molecules that influence the host physiological and immunological responses. Alternatively, the parasites may secrete factors that directly alter the host's hormone levels (Beckage, 1993).

The present study reports intersex and confirms the abnormality at microscopic level by the simultaneous presence of female and male germ cells in the same gonads. The intersex was exclusively observed in females heavily infested by nematodes, pointing out the probable impact of these parasites in the occurrence of horse mackerel female masculinisation.

MATERIAL AND METHODS

Study area and fish sampling

Two hundred and sixty-six specimens of *Trachurus trachurus* were randomly sampled from commercial fishing vessels from the Gulf of Bejaia, in the Eastern coast of Algeria. Sampling took place from February 2013 to March 2014. *T. trachurus* specimens were transferred to the laboratory and were immediately examined. Total lengths and weights of all specimens were measured to the nearest 0.1 cm and 0.1 g, respectively. The abdominal cavity of every fish was opened; sex was identified according to the external appearance of *T. trachurus* gonads (colour and tissue aspect) (Macer, 1974; Walsh *et al.,* 1990; Costa, 2001; Karlou-Riga, 2003).

Age estimation

Otolith extraction was performed through gill opening. It consisted in cutting the gill isthmus and in bending the head back away from the rest of the body; the prootic bulla were then carefully cracked and otoliths were extracted (Secor *et al.,* 1992). Otolith sagittae were mounted with drop of glue in glass slides, ground along the longitudinal plane using increasing granularity sandpaper (400 and 1200 grain). The exposed surface was then polished and smoothed using successively alumina paste and felt polishing. Finally, the glass slides were observed in a binocular microscope for otolith reading (Waldron and Kerstan, 2001).

Histological studies

A subsample of *T. trachurus* specimens was analyzed. As macroscopic intersex was observed in females highly infested by nematodes, individuals were chosen according to their infestation status. Five categories were considered: two infested females showing intersex macroscopically (white testicular component attached to ovarian tissue), nine infested females with no apparent intersex, three non-infested females with no apparent intersex, seven infested males with no apparent intersex and three non-infested males with no apparent intersex. These specimens were at the same reproduction stage (Walsh *et al*., 1990). Using Student t-test, no difference ($p < 0.05$) was observed between intersex individuals and the control group concerning total length, weight and age.

Gonads were fixed in 10% neutralized formalin and divided into equal pieces of transverse 5-mm thickness portions. These portions were then dehydrated through increasing ethanol concentrations (70-95%), cleared in xylene and embedded in paraffin. Histological sections of 3-µm thickness were then performed using a Leica RM2025 rotary microtome. Sections were mounted on glass microscope slides, stained with Mayer's haemalun-eosin, and mounted with Canada balsam. Samples were then examined by light microscopy and photographed using a digital camera.

Parasitological studies

Skin surface, fins, and gills were examined under stereomicroscope for attached parasites, lesions, or any other external changes (Morsy *et al.,* 2013). In order to detect parasitic worms, inspection of the internal organs (gonads, abdominal cavities, intestines, stomachs, livers) and muscles was performed using a stereomicroscope. For each fish, parasites were collected separately and the number of each parasite species was recorded. Light and scanning electronic microscopy (SEM) studies were performed on these larvae specimens in order to characterize their morphology according to Ichalal *et al*. (2015). For SEM analysis, nematodes (stored in 70% ethanol) were dehydrated in a graded series of ethanol: 80% (1/2 h), 90% (1/2 h), and 100% (1/2 h). The specimens were immersed in hexamethyldisilazane (HMDS: Molekula) for 1/2 h (twice). After removing the excess of HMDS, the specimens were allowed to evaporate overnight under a fume hood. Then, the samples were mounted on aluminium stubs (Agar Scientific) with double sticky carbon tabs (Agar Scientific) and sputter-coated under argon flow with Au/Pd Polaron SC 7620 for 90 s. They were examined in a SEM LEO 438 VP.

For each parasite, the following morphological characteristics were measured: body length and width, oesophagus length, ventriculus length, tail length, tail mucron, boring tooth and lips (Murata *et al.,* 2011). Then, nematodes were drawn using a camera Lucida for more morphological details in order to identify the species. Calculated parasitological indexes were: prevalence (P), which is the number of infected fishes divided by the number of examined fishes, expressed as a percentage; mean intensity (mI), representing the total number of parasites of a particular taxon divided by the number of infected fish; and mean abundance (mA), which is the total number of parasites of a particular taxon divided by the total number of examined fishes (Bush *et al.,* 1997).

RESULTS

Length, weight and otolith reading

Total lengths and weights of intersex specimens were ranged between 170-250 mm and between 36.2 and 121.4 g, respectively. Otolith reading revealed that the individuals were 1 to 5 years old.

Macroscopic examination of the reproductive system

The macroscopic intersex was observed in two females with a simultaneous presence of female and male gonads. Gonads were presented as testicular white components embedded in one of the two ovaries (Fig. 1). The testis components were not uniform in size or in location among the two intersex fishes and showed marked differences in their distribution patterns. The female portion represented the majority of the gonad tissue and showed pink pigmentation as in normal ovaries (Fig. 1). Macroscopic examination of external body showed that almost all specimens were free from important lesions and abnormalities.

Histological analysis

The histological analysis revealed that individuals showing macroscopic intersex presented apparent male and female functional tissue (Fig. 2A, 2C). Testicular portions presented seminiferous tubules-like structures containing male germinal cells at different stages of spermatogenesis similar to those observed in normal males (Fig. 3B, 3C), except the fact that no mature spermatozoa were observed. No microscopic intersex was retrieved in infested or non-infested males (N = 10), and in non-infested females (N = 3), the histological analysis revealed normal spermatogenesis and oogenesis (Fig. 3).

The histological examination of the apparent normal females infested by nematodes (N = 9), revealed the presence of testicular germ cells disseminated in the ovarian tissue (Fig. 2B). The testicular tissue was located on the periphery of the gonad consisting of an epithelial tissue in lobular or cystic structures. A predominance of spermatogonia was observed compared to spermatocytes and spermatids (Fig. 2D). No mature spermatozoa were observed.

Parasitological analysis

The distribution and localization of the collected parasites in the fish bodies showed that nematodes were found encapsulated (coiled in a thin-walled cyst) or as free larvae located on the surface of the abdominal organs. The worms were retrieved from the gonads, livers, intestinal cavities and stomachs. The digenean parasites were collected from stomachs and intestinal cavities. The parasites were identified to the species level. The parasitological analysis revealed that the total population (n = 266 individuals) presented the following indexes: for nematodes [*Anisakis simplex* (Rudolphi, 1809) and *Hysterothylacium aduncum* (Rudolphi, 1802)], P = 31.20%, mI = 2.59 per fish and mA = 0.80. Concerning digenean parasites [*Monascus filiformis* (Rudolphi, 1819)], the values were: P = 13.15%, mI = 1.51 per fish; mA = 0.19. Parasitological indexes of the subsample concerned by the histological investigation showed that nematode infestation rate was higher than those observed for Digenea, with P = 66.66%, mI = 3.87.per fish and mA = 2.58 *versus* P = 12.5%, mI = 1.33 per fish mA = 0.16. No ectoparasite was detected in the analyzed fish. Table I summarizes the different fish categories concerned by the histological analysis with the corresponding parasitological indexes and dates of capture.

DISCUSSION

Horse mackerel, *T. trachurus*, a gonochoric species, is widely distributed through the Mediterranean Sea (Gordo *et al*., 2008). Horse mackerel reproduction (Macer, 1974; Karlou-Riga and Economidis, 1995; Costa, 2009; Gonçalves *et al*., 2009) and parasitism (Bartoli and Bray, 2004; Campbell, 2005; Faye and Toguebayek, 2005; Abaunza *et al.,* 2008; MacKenzie *et al.,* 2008; Mattiucci *et al.,* 2008; Tantanasi *et al.,* 2012; Mansour *et al.,* 2013) have been extensively studied.

The present report revealed intersex in *T. trachurus* individuals with a simultaneous presence of male and female gonads. This macroscopic intersex represented 0.75% of the total collected individuals, a percentage lower than those reported for many fish species. Bernet *et al.* (2004), Bittner *et al.* (2009), Hinck *et al.* (2009), Djoudad-Kadji *et al.* (2012) reported 1.1%, 1.7%, 3% and 2.28%, respectively. However, microscopic intersex was found at much more frequency, in fact, 100% of apparent normal females but highly infested by nematodes (N = 9), were intersex specimens. These results are similar to those reported by Hinck *et al.*, (2009) in *Micropterus salmoides* (Lacepède, 1802) (8-91%) and *M. dolomieu* (Lacepède, 1802) (14-73%). Lower frequencies (25%) were also reported concerning *Zoarces viviparous* (Linnaeus, 1758) (Gercken and Sordyl, 2002) and *Xiphias gladius* (Linnaeus, 1758) (De Metrio *et al.*, 2003).

The mechanisms causing intersex remain not well understood (Devlin and Nagahama, 2002; Hinck *et al.*, 2009). However, many factors including exogenous steroids, temperature, pH, behavioural cues and pollutants are involved in sex differentiation in fish (Minier *et al.,* 2000; Devlin and Nagahama, 2002).

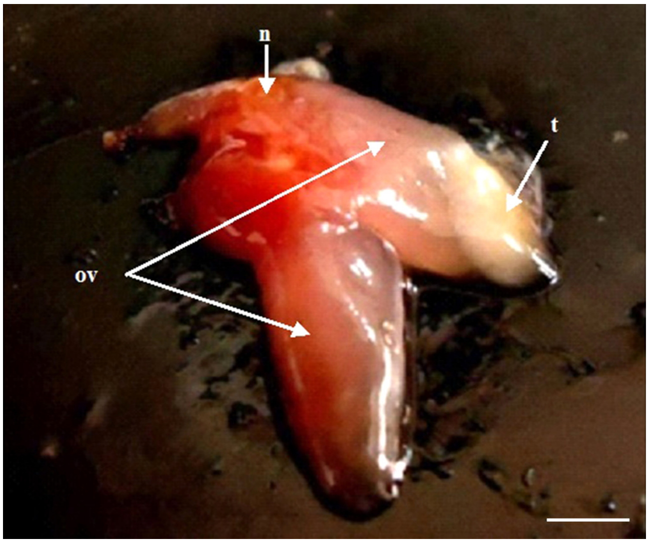

Figure 1. – Gonad of *Trachurus trachurus* identified as intersex, showing one abnormal form: portion of one small testis portion entirely linked to the ovary. n: nematodes; ov: ovary; t: testis. Scale bar = 1 cm.

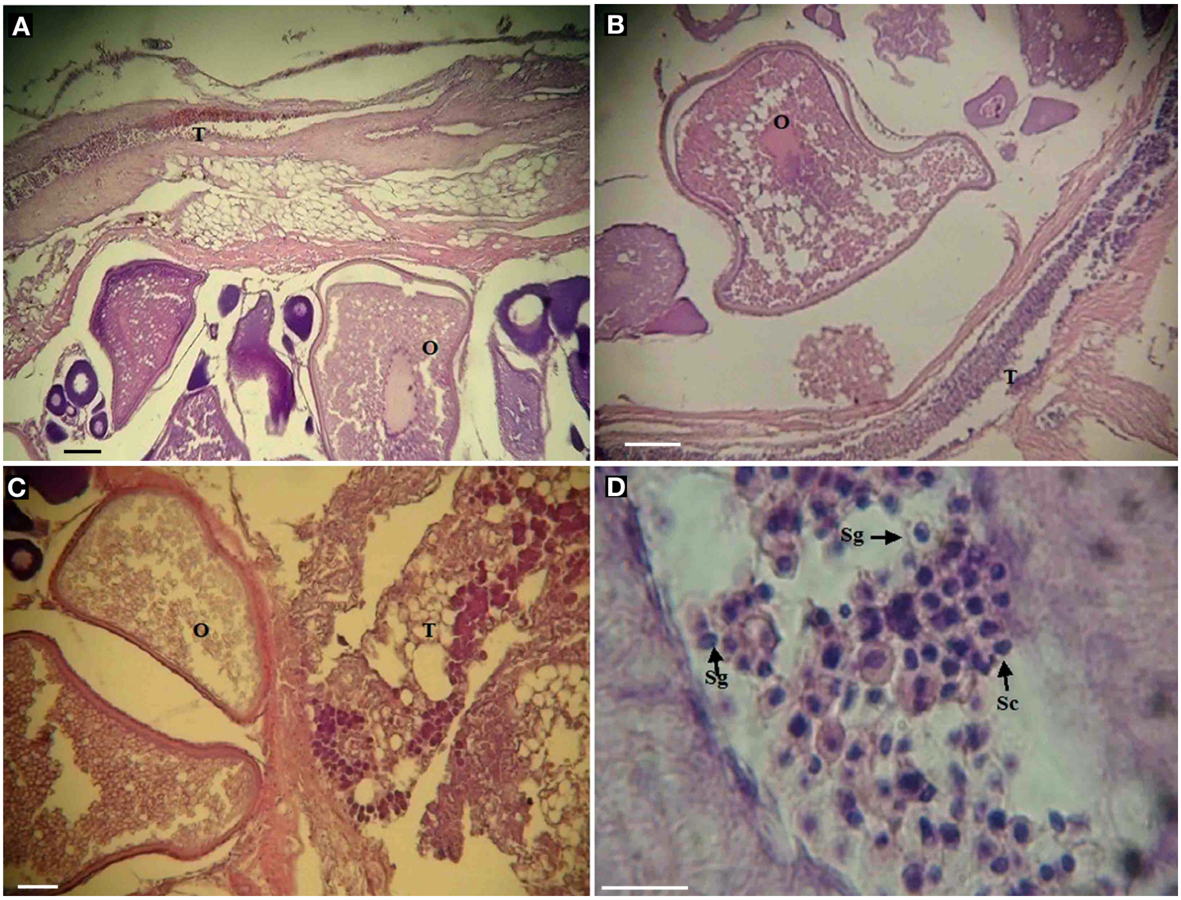

Figure 2. – **A**, **B**, **C**: Histological sections in intersex *Trachurus trachurus*; O: ovarian tissues; T: testicular tissues. **D**: Cells present in the male portion of intersex individuals; Sc: spermatocyte; Sg: spermatogonia. Scale bars: A, B, C = 100 μm; D = 20 μm.

In the current results, intersex was associated with nematode infestation; in fact, intersex was exclusively found in infested females. Previously, Schärer and Vizoso (2003) reported that parasites accelerate sex changes in fish naturally hermaphrodite. The authors confirmed that *Thalassoma bifasciatum* (Bloch, 1791) infected females with myxozoan *Kudoa ovivora* (Swearer & Robertson, 1999) have a lower reproductive success and change sex earlier at a smaller size than non-infected females. In the same context, Wiklund *et al*. (1996) have suggested that intersex found in Finnish roach could be caused by *Pleistophora mirandellae* (Vaney & Conte, 1901*)*, a microsporidian parasite.

In other animal species, parasitism is established as a direct inducer of sex reversal. Rodgers-Gray *et al*. (2004) reported that microsporidian parasite induces feminization by preventing androgenic gland differentiation in amphipods. Intersexuality was also observed in zooplankton *Mysid siriella* (Linnaeus, 1758) infected by the nematode ellobiopsilids (Ohtsuka *et al.,* 2003). However, parasitism-intersex association has been mostly explored in insects where nematodes have been reported by several authors as triggers of sex reversal (Wülker, 1975; Gordon, 1981; Vance, 1996; Rempel, 2005).

The present results seem to be in accordance with the previous reports suspecting parasites in fish physiologic (Schärer and Vizoso, 2003) and pathologic intersex (Wiklund *et al*., 1996). The mechanisms underlying sex reversal in relation to parasitological status remain not understood. Nevertheless, reports suggest that parasites could interfere with endocrine system at hormonal level (Beckage, 1993; Jobling and Tyler, 2003), in this respect, Arme (1997) and Minier *et al*. (2000) indicated that gonad development in parasitized fish remains poor and occasionally non-existent related to the interference of Nematoda with the host endocrine system. Some parasites, like cestode *Ligula intestinalis* (Linnaeus, 1758) (Carter *et al.,* 2005) and Nematoda *Eustrongylides* sp. larvae (Mir *et al.,* 2012), have been shown to interfere high in the pituitary-gonadal axis of the host, decreasing consequently the secretion of gonadotrophins. All these hormonal disruptions, known to be the key factor in intersex onset (De Metrio *et al*., 2003; Bahamonde *et al*., 2015 a, b; Guellard *et al*., 2015), could be involved in *T. trachurus* individuals expressing intersex.

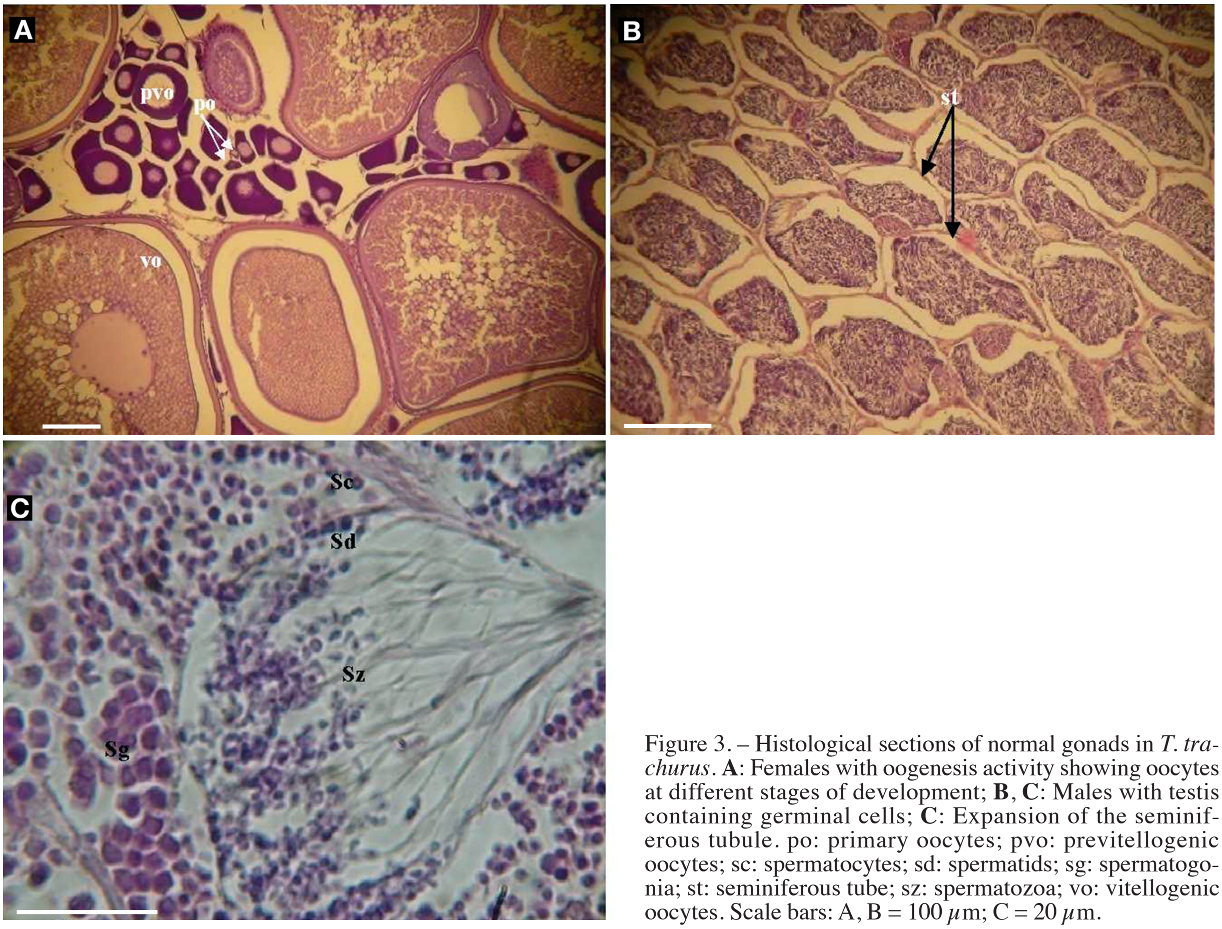

Figure 3. – Histological sections of normal gonads in *T. trachurus*. **A**: Females with oogenesis activity showing oocytes at different stages of development; **B**, **C**: Males with testis containing germinal cells; **C**: Expansion of the seminiferous tubule. po: primary oocytes; pvo: previtellogenic oocytes; sc: spermatocytes; sd: spermatids; sg: spermatogonia; st: seminiferous tube; sz: spermatozoa; vo: vitellogenic oocytes. Scale bars: A, B = 100 μm; C = 20 μm.

In fish, intersexuality is also significantly affected by the environment pollution (Jones and Reynolds, 1997; Parks *et al*., 2001; Van Aerle *et al*., 2001; Lokman *et al*., 2002; Gibson *et al*., 2005; Kipfer *et al*., 2009; Prado *et al*., 2011; Sanchez *et al*., 2011; Baynes *et al*., 2012; Blazer *et al*., 2012). It has been reported particularly, that heavy metals pollution impact negatively the reproductive function in fish (Popek *et al.,* 2006; Sun and Tsai, 2009; Ebrahimi and Taherianfard, 2011) including intersex induction (Sun and Tsai, 2009). In the same respect, zinc is involved in the masculinisation of the Equilateral Venus, *Gomphina veneriformis* (Lamarck, 1818) in aquatic environment (Ju *et al.,* 2009).

The Mediterranean Sea is among the busiest world waterways, and shipping traffic is continuously growing along with the concern for its potential impacts on marine fauna (Notarbartolo di Sciara and Birkun, 2010; Geijer and Jones, 2015). In addition, Mediterranean Sea is threatened by numerous factors, mainly wastewaters release and chemical pollution (Halpern *et al.,* 2008). In this respect, the Gulf of Bejaia, the location from which *T. trachurus* specimens were captured, presents several pollution sources including domestic waste, chemical and petrochemical industrial discharges, heavy metals, hydrocarbons and organic compounds (Grimes, 2003). Nematodes, being pollution accumulators (Mackenzie *et al*., 2008; Morsy *et al*., 2012), could be the mean factors in *T. trachurus* intersex onset.

In conclusion, the present results reported the first cases of intersex in *T. trachurus;* this condition was exclusively observed in females highly infested by Nematodes. Further studies are required to deeply understand parasite-pituitary-gonadal axis interaction, particularly in relation to the environmental pollution.

Table I. - Parasitological indexes according to fish categories. P: prevalence; mI: mean intensity; mA: mean abundance; N: Nematoda; D: Digenea.

Category of fish	Date of capture	Parasitological indexes					
		P%		mI		mA	
		N	D	N	D	N	D
Infested females expressing macroscopic intersex	22 December 2013 04 February 2014	100	0	1	0	1	0
Infested females with no apparent intersex	24 February 2013 26 March 2013 29 April 2013 06 June 2013 29 October 2013 20 November 2013 22 December 2013	100	22.22	2.44	1.5	2.44	0.33
Non-infested females with no apparent intersex (control group)	26 March 2013 08 October 2013 19 January 2014	0	0	0	0	0	0
Infested males with no apparent intersex (control group)	26 March 2013 29 April 2013 06 June 2013 29 October 2013 22 December 2013	85.71	14.28	6.5	1	5.57	0.14
Non-infested males with no apparent intersex (control group)	24 February 2013 20 November 2013 19 January 2014	0	0	0	0	0	0

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